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A Homogeneous, Fluorescence Polarization Assay for Src-Family Tyrosine Kinases

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A nonradioactive, simple, sensitive fluorescence polarization assay was developed to assay protein tyrosine kinase activity. This assay involves incubation of a fluorescenylated peptide substrate with the kinase, ATP, and anti-phosphotyrosine antibody. The phosphorylated peptide product is immunocomplexed with the anti-phosphotyrosine antibody resulting in an increase in the polarization signal as measured in a fluorescence polarization analyzer. Among several antiph sphotyrosine antibodies examined, monoclonal antibody PY54 was found to give the best polarization signal with the test peptide. For validation of the fluorescence polarization assay, Lck activity was compared with a $^{32}PO_4$ transfer assay. In both the fluorescence polarization and $^{32}PO_4$ transfer assays, Lck activity showed a similar dependence on ATP, Lck enzyme, and peptide substrate concentrations. Both assays gave similar inhibition constants with a known tyrosine kinase inhibitor staurosporine and the Lck inhibitor, 4-amino-5-(methylphenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]pyrimidine. These results show that the fluorescence polarization assay can detect inhibitors and is comparable to the $^{32}PO_4$ transfer assay. The fluorescence polarization method is advantageous compared to the $^{32}PO_4$ transfer assay or ELISA or DELFIA because it is a one-step assay that does not involve several washings, liquid transfer, and sample preparation steps. It has the added advantage of using nonisotopic substrates. The fluorescence polarization assay thus is environmentally safe and minimizes handling problems. The homogeneous nature of the assay makes it readily adaptable to high-throughput screening for small-molecule drug discovery. © 1997 Academic Press

Protein tyrosine kinases[2] (PTKs) play critical roles in the regulation of many cellular functions including differentiation, growth, and metabolism (1). Aberrations in signaling pathways can result in numerous diseases such as cancer, diabetes, atherosclerosis, inflammatory diseases, and immunological disorders (2–5). PTKs are a diverse group of protein kinases that include receptor PTKs, which are transmembrane proteins and nonreceptor cytoplasmic PTKs. Src-family PTKs play important roles in the regulation of growth, proliferation, and differentiation of many types of cells (6). Hence, developing a simple, reliable PTK assay to screen compound collections for inhibitors is an essential step in developing drugs for various diseases.

In the conventional radioactive PO_4 transfer protein kinase assay the radioactive phosphate from [γ-^{32}P] or [γ-^{33}P]ATP is incorporated into a protein/peptide substrate and is measured by binding the protein/peptide to phosphocellulose (P-81) filter discs or precipitating with TCA. After washing several times the product is counted in a liquid scintillation counter (7, 8). In addition to generating large volumes of radioactive waste, these steps cannot be completely automated, and the use of acid for washes corrodes and can clog liquid delivery systems. Solid-phase radioactive phosphate transfer assay in which the substrate (peptide/protein) is bound to a ScintiStrip micro plate (Wallac Inc.) or a Flash plate (Dupont NEN) that is coated with scintillant is also used for high-throughput screening (9). This approach has relatively fewer steps. The disadvantages of this method are the use of radioactive compounds generating radioactive waste, and a low signal to noise ratio. Alternative nonisotopic ELISA-type assays consist of coating a microtiter plate with the peptide substrate, washing excess reactants after the kinase assay,

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[2] Abbreviations used: PTK, protein tyrosine kinase; FP, fluorescence polarization; PY, phosphotyrosine; GST, glutathione-*S*-transferase; Hepes, 4-(2-hydroxyethyl)-l-piperazineethanesulfonic acid; Mops, 3-[*N*-morpholino] propanesulfonic acid; LSC, liquid scintillation counting; TCA, trichloroacetic acid; ELISA, enzyme-linked immunosorbent assay; DELFIA, dissociation-enhanced lanthanide fluorescence immunoassay; mP, milli-polarization units; DTT, dithiothreitol; PP, 4-amino-5-(methylphenyl)-7-(*t*-butyl)pyrazolo [3,4-*d*]-pyrimidine.

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incubating with a phosphotyrosine antibody and washing, followed by a secondary antibody conjugated to alkaline phosphatase or horseradish peroxidase, and a final incubation with the color-developing reagents (10–14). Though this ELISA-type assay can be automated, this method involves several washes, liquid transfers, and incubation times, and hence is very labor-intensive and time consuming. Recently, a sensitive, microtiter plate-based assay using time-resolved fluorometry of Europium chelate was described (15). This assay is similar to the ELISA method, uses Eu^{3+}-phosphotyrosine antibody, and eliminates incubation with the secondary antibody and color development. However, this approach still has many steps, has a high background, and is time consuming.

In an attempt to develop a simple alternative nonradioactive PTK assay, wherein all the reagents are combined in a single step and the signal is directly measured without additional processing steps, fluorescence polarization (FP) was evaluated. In 1926 Perin first described the utility of FP to study the molecular interactions in solution (16). FP is a powerful technology for the determination of molecular interactions in solution (16, 17). FP is determined as a ratio of the fluorescence emissions measured in the vertical and horizontal planes. FP (P) is calculated by the equation $P = (I_v - I_h)/(I_v + I_h)$, where I_v and I_h are the fluorescence vertical and horizontal intensities, respectively. When fluorescent molecules in solution are excited with polarized light, the degree to which the emitted light retains polarization reflects the rotation that the chromophore underwent during the interval between absorbance and reemission. If the molecules are small, they rotate or tumble faster and the resulting emitted light is random with respect to the plane of polarization (depolarized). If small molecules bind to large molecules, the fluorescent molecules can remain relatively stationary and the emitted light will retain a proportional degree of polarization that is seen as an increase in the FP signal. FP has been used extensively in the last decade in competitive immunoassays for the detection of the drugs and hormones (18, 19). The applications of FP have been extended to protein–DNA interactions (20), protease and nuclease assays (21), DNA hybridizations (22), protein–protein interactions (23), and receptor–ligand binding.

To develop a FP assay for a soluble PTK, we used Lck which is a member of the Src family of cytoplasmic nonreceptor PTKs and is essential for the development of T cells and the activation of T cells by antigen (24). We describe here a very simple one-step, nonradioactive FP assay for Lck.

MATERIALS AND METHODS

The recombinant mouse Lck fused to GST was expressed in Sf9 baculovirus and purified, and was kindly provided by Dr. J. Fargnoli (Biomolecular Drug Discovery, Bristol-Myers Squibb). The purified GST-Lck (Lck), at 1 mg/ml, was aliquoted and stored at −80°C. Lck peptide substrate Ala-Glu-Glu-Glu-Ile-Tyr-Gly-Val-Leu-Phe-Ala-Lys-Lys-Lys-Lys tagged with fluorescein either at the amino (peptide A) or carboxyl terminus (peptide B) was synthesized by Research Genetics. Monoclonal anti-phosphotyrosine (PY) antibodies PY20, PY54, and PY69 and rabbit anti-PY polyclonal antibody were obtained from Transduction Labs; PY 4G-10 antibody was obtained from Upstate Biotechnology Inc. Staurosporine was from LC Laboratories. The Lck inhibitor (25), 4-amino-5-(methylphenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]pyrimidine (PP), was synthesized in the Chemistry Department at Bristol-Myers Squibb (Princeton, NJ). [γ-^{32}P]ATP was from NEN-Dupont. All the other chemicals used were of highest purity and purchased from Sigma.

Fluorescence polarization assay. The reaction mixture, consisting of peptide substrate (0.45–9.5 μM), Lck (0.5–2 μg/ml), ATP (0.01–0.5 mM), and anti-PY antibody (5–20 μg) in assay buffer (50 mM Hepes, 15 mM $MgCl_2$, 5 mM $MnCl_2$, and 1 mM DTT, pH 7) at a final volume of 100 μl in a 12 × 70-mm borosilicate glass tube, was incubated at 25°C. When inhibitors were used, 10 mM stock solutions were made in DMSO, diluted fresh in the assay buffer, and added to the reaction. The assay buffer was modified in some assays to contain 20 mM Mops and 10 mM $MgCl_2$, pH 7. Following a 2-h incubation, the assay mixture was diluted to 2 ml with 20 mM Hepes or Mops (pH 7) and FP was measured in a FPM-1 fluorescence polarization analyzer (Jolley Consulting and Research Inc.). FPM-1 is a single-cell instrument and the volume required for measurement is a minimum of 1 ml. The polarization value, P, is a ratio of light intensities and is expressed in millipolarization units (1 polarization unit = 1000 mP units). In the time–course reactions, the reaction was stopped with the addition of 2 ml of 20 mM MOPS with 20 mM EDTA (pH 7) at the times indicated and the FP signal was measured.[3]

$^{32}PO_4$ transfer assay by P-81 filter paper assay. For validation of the FP-Lck assay, Lck activity by FP was compared with the enzyme activity assayed by $^{32}PO_4$ transfer from [γ-^{32}P]ATP. The assay mixture was the same as that used in the FP-assay without PY antibody, but containing [γ-^{32}P]ATP as a tracer (~500 cpm/pmol). Following incubation, 50 μl of the reaction mixture was spotted onto a 1.5-cm-square P-81 disc (What-

[3] In the initial assay reactions at the end of incubation, the reactions were diluted with 20 mM Mops, pH 7, and read in a FP analyzer. In the later assays, the samples were routinely diluted with 20 mM Mops, pH 7, containing 20 mM EDTA. It is recommended to dilute the reaction with 20 mM Mops, pH 7, containing 20 mM EDTA, which terminates the reaction before measuring the FP signal.

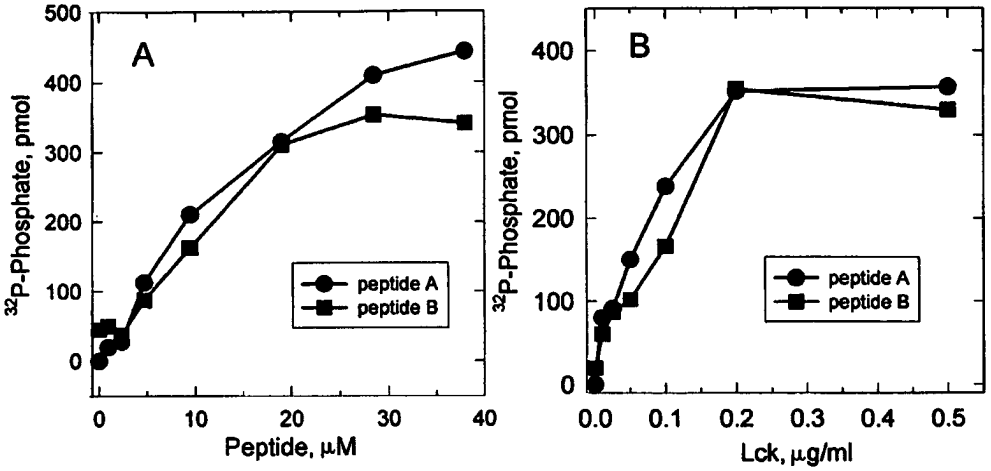

FIG. 1. Comparison of peptide A and peptide B as substrates for Lck tyrosine kinase. $^{32}PO_4$ incorporation into peptide A and peptide B was measured by P-81 filter paper method. (A) The dependence of Lck activity on peptide concentration was studied by increasing peptide concentration at 1 μg/ml Lck. (B) The dependence of Lck activity on Lck concentration at 9.47 μM peptide.

man Co.) and immediately immersed into a wire mesh basket placed in a beaker of 75 mM phosphoric acid (8). The filters were swirled gently for 5 min, the phosphoric acid was decanted, and the filters were then washed three times in phosphoric acid with gentle agitation to remove any unreacted [^{32}P]ATP. After the washes, the filter discs were blotted between Whatman 3 filter papers, air dried, and transferred into vials containing 3 ml scintillation fluid and the radioactivity was measured in a beta scintillation counter (Packard).

RESULTS AND DISCUSSION

Fluorescein-labeled synthetic peptide as substrate for Lck. The PTK used in developing the FP assay was a recombinant GST-fused Lck. Optimal peptide substrate sequences recognized by different PTKs based on the phosphorylation sites of natural substrates or by using an oriented peptide library were described (26, 27). A peptide substrate Ala-Glu-Glu-Glu-Ile-Tyr-Gly-Val-Leu-Phe-Ala-Lys-Lys-Lys-Lys for Lck with either the N-terminal alanine (peptide A) or the C-terminal lysine (peptide B) labeled with fluorescein was synthesized. To evaluate the efficacy of peptide A and peptide B as substrates for Lck, phosphorylation of these substrates by [^{32}P]ATP was measured in a P-81 filter assay (see Materials and Methods). Both substrates were phosphorylated to approximately the same degree and gave similar substrate saturation curves with K_m's of 18.5 and 19 μM for peptides A and B, respectively, compared to 16.6 μM for nonfluorescent peptide (Fig. 1). These K_m's are within the range (5 μM to 1 mM) noted for synthetic peptides as substrates for PTKs (28). Dependence of substrate phosphorylation on Lck enzyme concentration was studied with 9.5 μM of peptide substrate (Fig. 1B). Phosphorylation of both peptides A and B was similar, increased linearly from 0.1 to 2 μg/ml Lck, and plateaued at higher concentrations. These results show that the two fluorescein-labeled peptides synthesized are essentially identical in their affinity as substrates of Lck. Peptide A was used as substrate in the subsequent FP assays.

Antibody preference in the FP assay. In the FP-Lck assay the product formed, phosphorylated fluorescein-labeled peptide A, is immunocomplexed to anti-PY antibody. The small fluorescent peptide substrate, which has free rotational mobility, is converted to a larger fluorescent phosphopeptide–antibody complex with restricted rotational mobility. Upon antibody binding, light emitted by the antibody bound peptide will retain a greater degree of polarization. Substrate (unphosphorylated peptide) will not bind antibody, and an increase in polarization should, in principle, allow one to monitor the reaction's progress. In the initial assays employing the very commonly used anti-phosphotyrosine antibody PY20, the FP signal obtained was marginal. To determine if different PY antibodies bind phosphorylated peptide A with different affinities and/or capacities to immobilize the phosphorylated peptide, several commercially available anti-PY antibodies that differed in IgG subtypes, PY20 (IgG2b), PY54 (IgG1), PY69 (IgG2a), PY4G10 (IgG2b$_\kappa$), and polyclonal rabbit anti-PY antibody, were evaluated in the FP assay. PY54 antibody gave the highest polarization signal which was about 100 mP above the control (Fig. 2). The polyclonal antibody and PY4G10 were the least effective. These results show that the PY antibodies of different IgG subclasses have different affinities to the tyrosine-phosphorylated peptide.

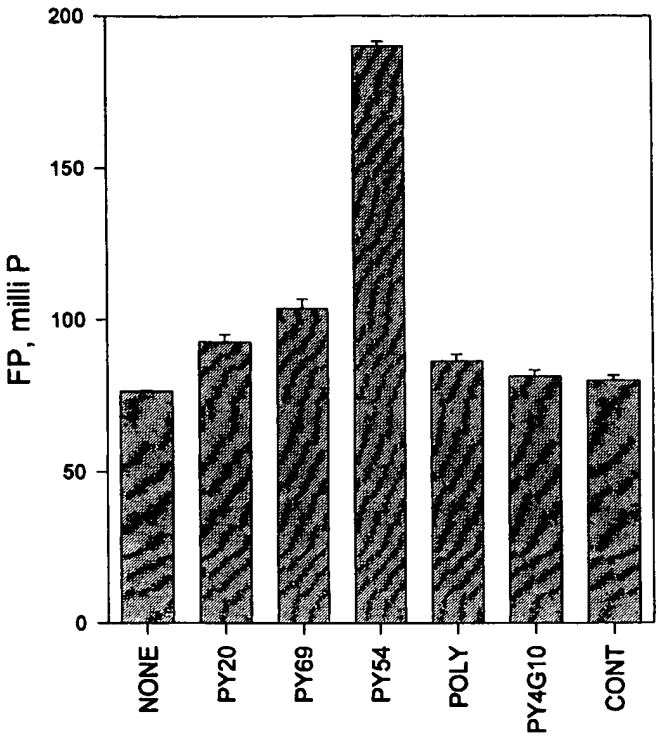

FIG. 2. Comparison of different PY antibodies for Lck activity by FP method. Lck activity was assayed for 2 h with 2.37 μM peptide A, 1 μg/ml Lck, and 0.1 mM ATP with 5 μg of each of the different PY antibodies.

To demonstrate that the FP signal is a result of binding of the phosphorylated peptide A to the antibody, the Lck assay was performed as a function of peptide A concentrations with 5 μg of PY54 in the assay. At 0.45 μM of peptide A and 5 μg of PY54 the FP signal increased from 70 to 177 mP. At higher concentrations of peptide A, the FP signal decreased, though the product formed increased linearly at these below K_m substrate concentrations. If PY54 was limiting, addition of more PY antibody would make more antibody available for complexing with the phosphorylated peptide. In fact, following the addition of another 5 μg of PY54 to the reaction containing 2.37 μM of peptide A the FP signal increased. Similarly, at 4.7, 7.1, and 9.5 μM peptide A concentrations, addition of 20 μg of additional PY54 restored the signal to about 170 mP (Fig. 3A). Though the product formed increased with increasing peptide concentrations, the ratio of phosphorylated peptide (product) to substrate (nonphosphorylated peptide) remains about the same (0.22), and the FP signal in the presence of excess antibody therefore also remains constant under these experimental conditions (about 170 mP). These data show that the FP signal is ratiometric and dependent on the PY antibody immunocomplexing to the product (phosphorylated peptide A). Addition of PY antibody at the beginning of the assay or at the end of the assay did not have any effect on the FP signal. To further demonstrate that the FP signal depends on the immunocomplex formation of phosphopeptide with PY antibody, increasing phosphopeptide amounts that approximated the product formed (see Fig. 3 legend) at the peptide concentrations used in Fig. 3A were used for immunocomplexing with 5 μg of PY 54. Figure 3B shows that excess phosphopeptide at limited PY antibody results in a decreased FP signal which can be restored by the addition of more antibody as expected, paralleling the results seen in Fig. 3A. The issue of substrate dependence is discussed below.

Dependency of the FP signal on the PY antibody was also demonstrated in an experiment in which 9.5 μM of peptide A in the FP assay was titrated with PY54. In this experiment the FP signal continued to ris up to 20 μg of PY54 (Fig. 4). In the Lck assay typically about 15–20% of peptide A was phosphorylated (determined by the $^{32}PO_4$ incorporation), and assuming 2 mol of antigen binding to IgG molecule in immunocomplex formation, about 25 μg of PY antibody is required to complex the product formed with 20 μM substrate, which agrees with the experimental results.

Time course of FP assay. To determine the optimal conditions for the FP assay, the time dependence of FP signal appearance (substrate phosphorylation) was studied with various concentrations of the substrate (Fig. 5B) at 50 ng Lck and 100 μM ATP. The FP signal with 1 μM peptide A increased rapidly and reached a maximum (230 mP) in about 90 min; as the concentration of the peptide increased, the rate of increase in the FP signal decreased, and the time required for reaching the maximum signal increased. At higher substrate concentrations the absolute amount of product formed increased while the ratio of product to substrate decreased; hence, the FP signal is lower at the same time point as the substrate concentration increased. The time taken to reach maximum product to substrate ratio (equilibrium) increased with increasing substrate concentration. Because the FP signal is not dependent on the absolute amount of product formed, at a given time point, the FP signal decreases with increasing substrate concentration if the enzyme is limiting. This is quite contrary to the other type of assays that measure the amounts of product. In these more standard reactions a good correlation is seen between the product formed and substrate until saturation is reached. When the enzyme and other reactants are in excess, the equilibrium was reached much more rapidly (Fig. 5A) than when they were limiting (Fig. 5B). From 1 to 10 μM peptide A, at 500 ng Lck and 200 μM ATP, the maximum FP value was reached within 10 min and at 15 and 20 μM peptide the FP value was maximum at 15 and 30 min, respectively. In a different method of

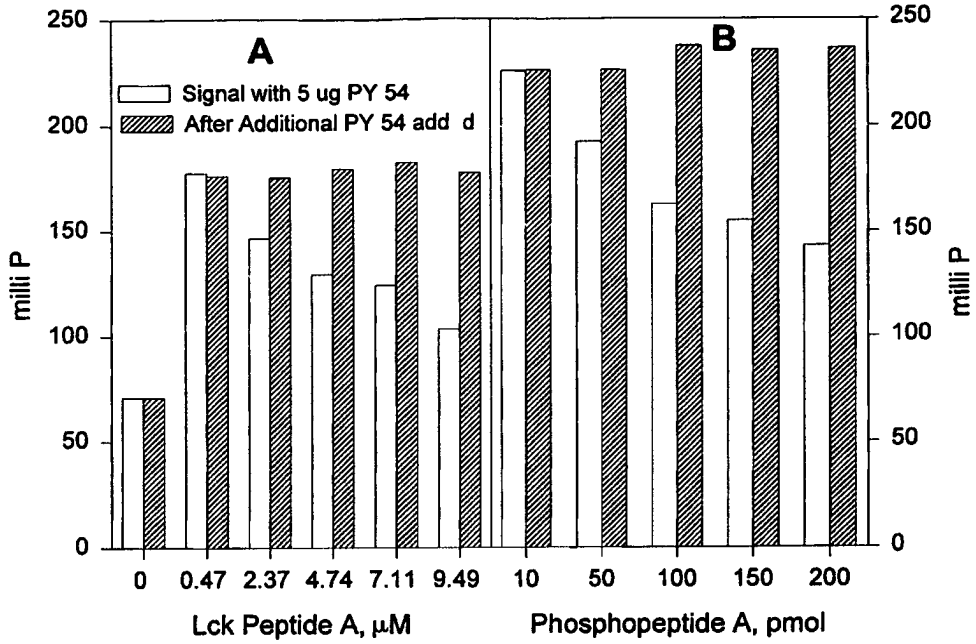

FIG. 3. Dependence of FP signal on antibody concentration. (A) FP assay was performed with increasing peptide A concentration with 5 μg of PY 54. FP signal was measured after 2 h incubation (clear portion of the bar). Additional PY 54, 5 μg for 0.47 and 2.37 μM peptide A reactions and 20 μg for 4.74, 7.11, and 9.49 μM peptide A reactions, was added and FP signal was measured after 30 min incubation. The increased FP signal due to the second addition of PY 54 is shown by the hatched area of the bar. (B) FP signal response at different concentrations of phosphorylated fluorescent peptide with PY 54. At the peptide A concentrations of 0.47, 2.37, 4.7, 7.1, and 9.5 μM used in the Lck reaction, about 9, 53, 107, 156, and 207 pmol of phosphate were transferred, respectively (in 100 μl reaction) in the $^{32}PO_4$ transfer assay. To demonstrate that the FP signal is due to immunocomplex formation between phosphorylated peptide A and antibody, 10, 50, 100, 150, and 200 pmol of synthetically phosphorylated peptide A were incubated initially with 5 μg antibody and the FP signal was measured after 30 min incubation. FP signal after adding additional 5 μg of PY 54 for 10 and 50 pmol and 20 μg of PY 54 for 100, 150, and 200 pmol is shown by hatched bars. Note that the FP signal is higher (230–240 mP) compared to FP signal in (A) because there is no contribution to the FP signal from the substrate, peptide A.

FP assay, a FP competition immunoassay, it is possible to measure the amounts of product formed.[4]

Evaluation of FP assay as a function of ATP, Lck, and peptide A concentration. In the FP assay, to detect the FP signal a substantial amount of substrate (at least 15%) has to be converted to product. Though the total fluorescence of the peptide substrate is peptide concentration dependent, the FP signal is not concentration dependent and is the same for 1 and 100 μM peptide A substrate. Theoretically, the minimum FP possible is 0 and the maximum is 500 mP for fluorescein. However, the real experimental minimum observed is 40–80 mP for small molecules, and the experimental maximum for large molecules is 100–200 mP. This window of FP signal between the minimum and maximum is sufficient because the ratiometric FP signal is highly reproducible. The FP signal is proportional to the phosphorylated peptide formed in the assay until it reaches maximum (170–200 mP) at a constant peptide concentration, if the PY antibody is in excess. Together, these requirements prescribe a narrow range of peptide substrate concentrations and antibody levels that can be used to operate a linear assay with respect to enzyme and ATP concentrations.

To validate the FP-Lck assay procedure, the FP assay was compared to a more standard protocol that measures the transfer of $^{32}PO_4$ from [γ-^{32}P]ATP as described under Materials and Methods. When peptide A (2.37 μM) phosphorylation was measured as a function of Lck concentration at 0.5 mM ATP, the polarization signal and $^{32}PO_4$ incorporated into peptide A increased rapidly and linearly up to 0.50 μg/ml Lck and reached saturation at higher concentrations (Fig. 6). As shown in Fig. 6A Lck activity measured by the FP assay and $^{32}PO_4$ transfer assay parallel one another very closely. The optimal concentration of 0.50 μg/ml Lck was used in the subsequent assays.

To determine the dependence of the Lck assay on ATP, the assay was performed varying the ATP concentration at 0.50 μg/ml Lck and 2.37 μM peptide A. Lck activity measured by both FP and $^{32}PO_4$ transfer assays increased sharply and reached saturation at 50 μM (Fig. 6B). The K_m for ATP was 2.5 and 3.3 μM in the $^{32}PO_4$ transfer and FP assay, respectively. Once again

[4] R. Seethala and R. Menzel, manuscript submitted for publication.

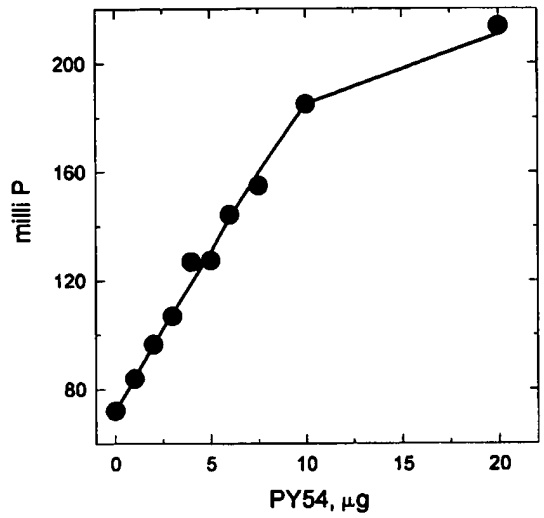

FIG. 4. PY54 titration of Lck. Lck activity by FP was assayed with 9.49 μM peptide A, 0.5 mM ATP, 2 μg/ml Lck, and increasing amounts of PY54 antibody.

the profiles of the FP signal and $^{32}PO_4$ incorporation are similar, showing that the FP-Lck and the $^{32}PO_4$ transfer assay gave comparable results.

The dependence of the FP signal on peptide concentration is complex because the FP signal is composed of contributions from both phosphorylated product and unphosphorylated substrate. This situation acquires an added degree of complexity if antibody is not in excess. The substrate saturation curve followed Michaelis–Menten kinetics in the $^{32}PO_4$-transfer assay with a K_m of 18.5 μM (Fig. 1). This means that the initial reaction is linear with substrate concentration until saturation is reached, and the percentage of peptide A converted to phosphorylated peptide is constant in this linear range of the substrate concentration. Because the FP signal is ratiometric, the signal is constant in the linear range of substrate concentration. In fact, the FP signal was 170 mP at lower substrate concentrations and started decreasing at higher substrate concentrations due to limiting PY antibody used (10 μg) in the assay (data not shown). Hence, this FP assay is not suitable for simple peptide substrate saturation studies as the FP signal is a measure of product to substrate ratio and is not dependent entirely on the absolute amount of product formed. A variation on this theme in a competition format in which nonfluorescent product competes with a fluorescent phosphorylated peptide tracer gives more readily interpretable peptide substrate saturation data.[4]

Inhibition of Lck activity by inhibitors. To determine if the FP assay will successfully detect inhibitors of Lck, staurosporine, a potent nonspecific protein kinase inhibitor, and PP, a specific Lck/Fyn inhibitor competitive with ATP (25), were evaluated simultaneously in FP and $^{32}PO_4$ transfer assay at 10 and 25 μM ATP. The inhibition curves were similar in both FP and $^{32}PO_4$ transfer assay (Fig. 7). The IC_{50} for staurosporine was 32.5 and 40.8 nM at 10 μM

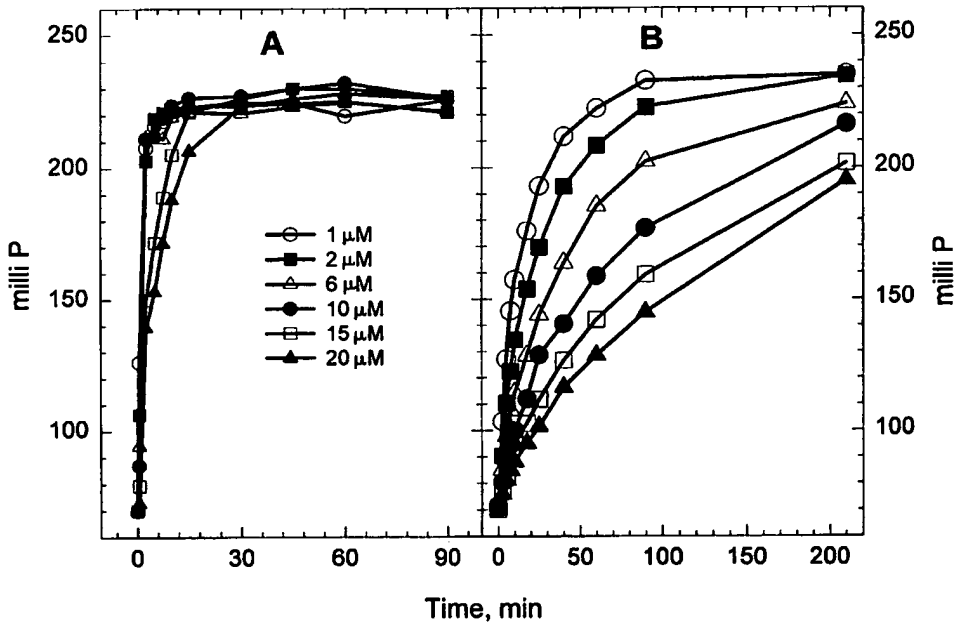

FIG. 5. The time dependence of FP signal was measured with increasing concentrations of peptide A and 10 μg PY54 (A) at 500 ng Lck and 200 μM ATP or (B) at 50 ng Lck and 100 μM ATP. The reaction was stopped with the addition of 2 ml of 20 mM Mops buffer, pH 7, containing 20 mM EDTA.

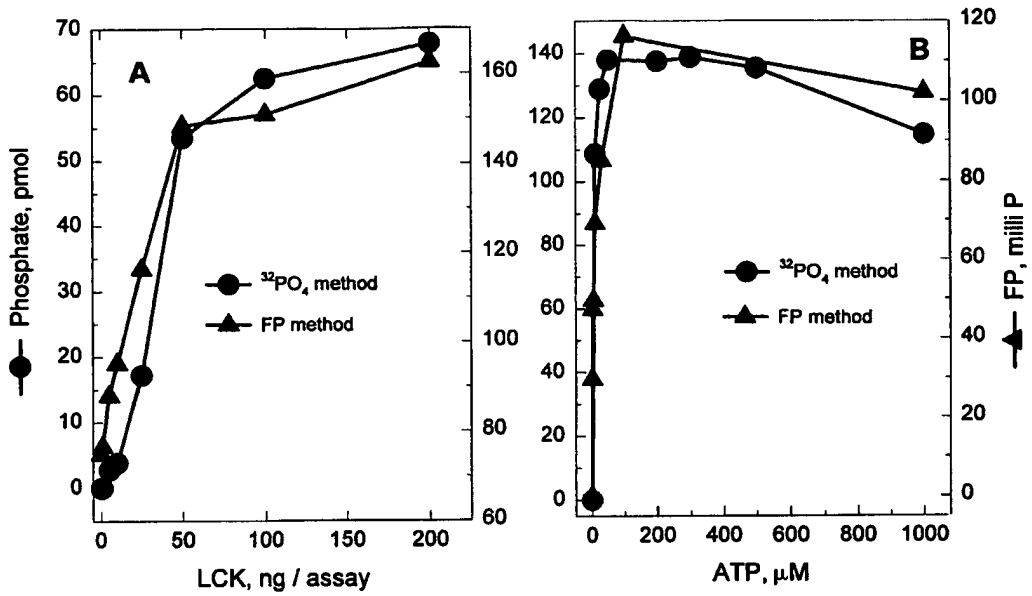

FIG. 6. Comparison of Lck activity in the FP and $^{32}PO_4$ transfer assay. (A) Lck activity was measured at 4.74 μM peptide A, 0.05 mM ATP, and 10 μg PY54, varying the enzyme concentration. (B) Lck activity was measured at 4.74 μM peptide A, 0.5 μg/ml Lck, and 10 μg PY54, varying ATP concentration. Note that the right axis in (B) mP values obtained after subtracting the blank FP value of peptide substrate.

ATP, and 120 and 140 nM at 25 μM ATP, in the FP and $^{32}PO_4$ transfer assays, respectively. IC_{50} for the Lck inhibitor PP was 0.21 and 0.07 μM at 10 μM ATP, and 0.40 and 0.25 μM at 25 μM ATP, in the FP and $^{32}PO_4$ transfer assays, respectively. The IC_{50} values for PP in the FP assay were two- to threefold higher than those determined with the $^{32}PO_4$ transfer assay. The antibiotic staurosporine is a potent inhibitor of Src family kinases (IC_{50} = 100–200 nM) (25) and the inhibition obtained with the peptide A substrate in this assay was within the expected submicromolar range (30–140 nM). With PP the IC_{50} values obtained with peptide A at relatively high ATP (10 and 25 μM) were lower (70–400 nM) than those reported (5–20 nM) using enolase as a substrate at low ATP concentrations (25). The substrate and ATP concentration differences easily explain the differences between these results and those previously reported. Nevertheless, these results showing similar inhibition with staurosporine and PP in the FP and

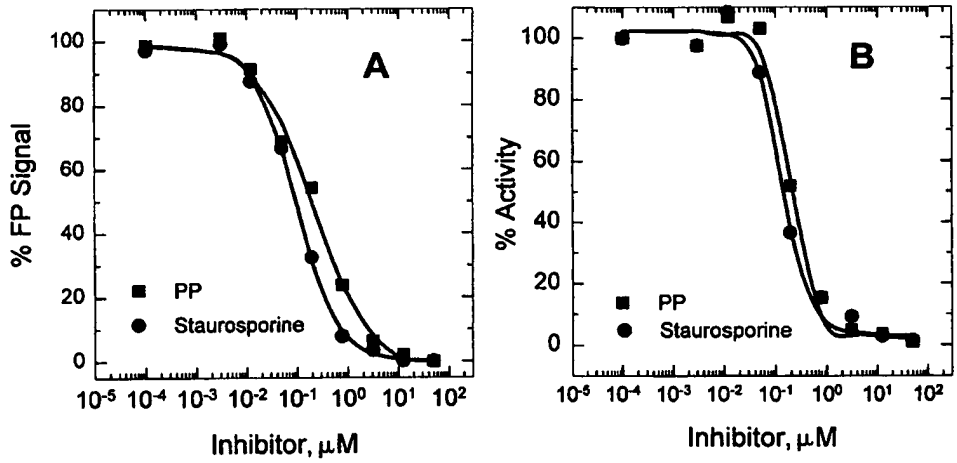

FIG. 7. Inhibition of Lck activity by staurosporine and PP in the FP (A) and $^{32}PO_4$ transfer (B) assay. Inhibition of Lck activity by increasing concentrations of inhibitors was measured at 4.74 μM peptide A, 0.025 mM ATP, and 15 μg PY54. The inhibition of Lck activity was also measured at 0.01 mM ATP (data not given).

TABLE 1

Comparison of Different PTK Assays

Fluorescence polarization	ELISA	Fluorometry	Radioactive PO_4 transfer assays	
			Liquid-phase	Solid-phase
1. *Kinase reaction* ATP + PY antibody read in fluorescence polarization analyzer	1. Coat plates with substrate, overnight 2. Wash 3. *Kinase reaction* ATP 4. Wash 5. Block (0.5–1 h) 6. Wash 7. PY antibody (0.5–1 h) 8. Wash 9. Secondary antibody (0.5–1 h) 10. Wash 11. Color development (15 min) 12. Stop color development 13. Read in spectrometer	1. Coat plates with substrate, overnight 2. Wash 3. *Kinase reaction* ATP 4. Wash 5. Block (0.5–1 h) 6. Wash 7. Eu^{3+}-PY antibody (0.5–1 h) 8. Wash 9. Enhancement solution (>5 min) 10. Read in fluorometer	1. *Kinase reaction* [^{32}P]ATP 2. Spot on P-81 filter discs 3. Wash and dry 4. Transfer to a vial 5. Add scintillation fluid 6. Count in a LSC	1. Coat ScintiPlates/ FlashPlates with substrate, overnight 2. Wash 3. *Kinase reaction* [^{33}P]ATP 4. Wash 5. Count in a LSC

$^{32}PO_4$ transfer assays, coupled with the results of enzyme and substrate dependence, show that the FP assay is a reliable alternative to the $^{32}PO_4$ transfer assay.

Comparison of FP assay with other kinase assays. In Table 1, the FP assay method is compared with various other methods. ELISA and DELFIA methods have too many processing steps (each additional step will reduce the sensitivity) which are time consuming. The radioactive phosphate transfer liquid and solid-phase assays have fewer steps than the ELISA and DELFIA assays and require the use of radioactive ATP, but are still more involved than FP. The FP assay is a very simple one-step method and is an excellent, alternative, nonradioactive method particularly appropriate for high-throughput screening of compounds for tyrosine kinase inhibitors. Some of the natural product or synthetic compounds used in screening have natural fluorescence, may absorb at the same wavelength as fluorescein, and may interfere in the FP assay. Interference by these compounds can be overcome by tagging peptides with alternative fluorescence dyes, such as rhodamine or Texas Red, that have different excitation and emission λ. The other disadvantage in this FP kinase assay is that the substrate has to be a small peptide and may not have as high an affinity as a native protein substrate. With a peptide substrate with high K_m, large amounts of phosphotyrosine antibody are needed to detect the FP signal. To overcome these problems we are evaluating an alternative FP competition immunoassay in which the phosphorylated substrate (substrate can be either a peptide–protein domain fused to GST or a protein) competes with fluorescent phosphopeptide tracer for anti-PY antibody which is similar to the established immunoassays.

The FP assay described here is a very simple, nonradioactive, and highly sensitive assay for PTKs. A variation of this method would also be suitable for the assay of phosphatases. The simplicity, speed, and homogeneous nature of this method make it ideal for high-throughput screening in small-molecule drug discovery programs.

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